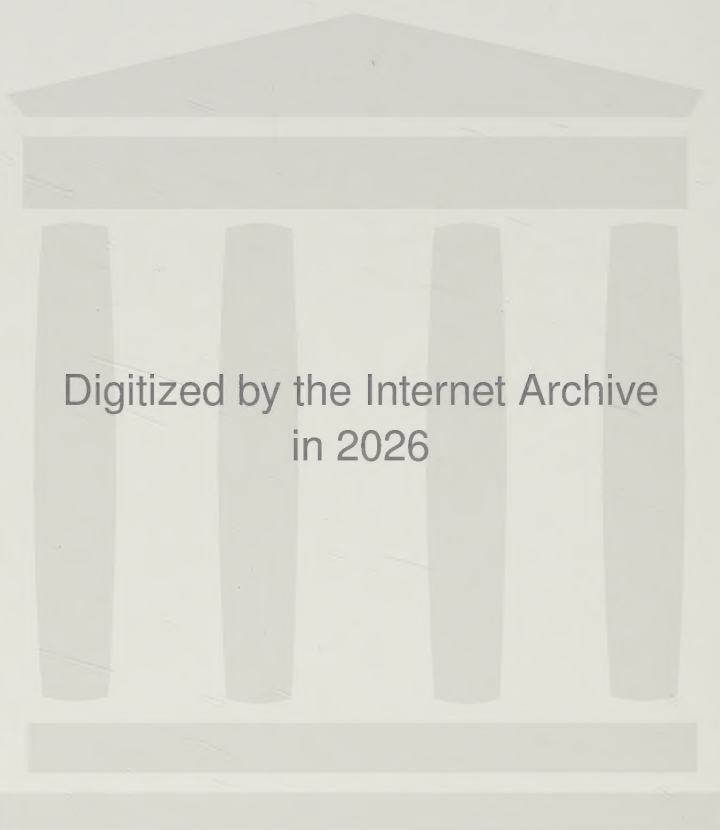


Claës-Otto Kindmark

C-REACTIVE PROTEIN

Malmö 1972



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C-REACTIVE PROTEIN

by

Claes-Otto Kindmark

Malmö 1972



Translated by L. James Brown

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- II. P.-O. Ganrot & C.-O. Kindmark: A simple two-step procedure for isolation of C-reactive protein. Biochim. biophys. Acta 194, 443, 1969.
- III. P.-O. Ganrot & C.-O. Kindmark: C-reactive protein - A phagocytosis-promoting factor. Scand. J. clin. Lab. Invest. 24, 215, 1969.
- IV. C.-O. Kindmark: Stimulating effect of C-reactive protein on phagocytosis of various species of pathogenic bacteria. Clin. exp. Immunol. 8, 941, 1971.
- V. C.-O. Kindmark: In vitro binding of human C-reactive protein by some pathogenic bacteria and zymosan. To appear in Clin. exp. Immunol., 1972.
- VI. C.-O. Kindmark: Identification of C-reactive protein by agarose gel electrophoresis of human serum or plasma. Clin. chim. Acta 35, 491, 1971.
- VII. C.-O. Kindmark & J.I. Thorell: Quantitative determination of individual serum proteins by electro-immuno-assay and use of ^{125}I -labelled antibodies (Application to C-reactive protein). To appear in Scand. J. clin. Lab. Invest. 29, Suppl. 124, 1972.
- VIII. C.-O. Kindmark: The concentration of C-reactive protein in sera from healthy individuals. To appear in Scand. J. clin. Lab. Invest. Vol. 29, 1972.

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PREVIOUS INVESTIGATIONS

C-REACTIVE PROTEIN

Discovery of CRP. About 40 years ago Tillet and Francis (125) found that when a certain carbohydrate fraction (Fraction C) of the cell wall from rough pneumococci was added to sera from patients with pneumonia it formed a precipitate. The precipitation was demonstrated with sera obtained very early in croupous pneumonia, i.e. after the acute stage, but not with sera obtained during convalescence, by which time the specific antibodies had appeared. The reaction was ascribed to the presence of a "C-reactive" substance in the sera. But the reaction was also demonstrable in sera from patients with diseases produced by gram-negative bacteria (8). Abernethy and Avery (1) later demonstrated that the C-reactive substance was a protein. It was therefore called C-reactive protein (CRP).

In the late 30s Löfström (67) showed that in human disease the serum often contains a substance capable of causing capsular swelling and agglutination of certain types of pneumococci. The substance was called "non-specific capsular swelling substance". In 1944 Löfström (69) produced convincing evidence that this substance was identical with CRP.

Preparation of CRP. MacLeod and Avery (70) were the first to publish a method for preparing highly purified CRP. They clarified the key-position of calcium ions in CRP-C-polysaccharide binding. They also showed that CRP was precipitated between 50-75 % saturation of ammonium sulphate. This method for

preparing human CRP, though with slight modifications, has long been used with success, and the preparations are pure enough to be crystallised (72).

Füvessy and Böszörményi (30) used a very different method for preparation of CRP i.e. precipitation with cold alcohol, in combination with precipitation with ammonium sulphate.

In 1963 Hokama and Riley (46) described a method for purifying CRP. Their method is based on ammonium sulphate precipitation followed by chromatography on DEAE-cellulose. The method has since been modified (42) by addition of rechromatography on DEAE-cellulose and gel filtration with Sephadex G-200.

On the basis of findings made by different groups (18, 37, 50) indicating that CRP was bound to phosphate groups via calcium, Riley and Coleman (1970) reported isolation of CRP by chromatography on phosphorylated cellulose (102).

Another method for preparing CRP- though not highly purified - was introduced by Davis et al. in 1971 (21). He used an immunoadsorbent.

Physical and chemical properties of CRP. The electrophoretic mobility of CRP is still debatable (for review see Schwarz (115) and Nilsson (84)). Some authors believe CRP to occur in the α , β or γ -region. Recently Hornung and Frichi (51) reported an interindividual variation of the mobility of CRP in serum.

According to Gotschlich and Edelman (36) CRP has a molecular weight of 110,000 to 144,000 and consists of an aggregate of probably 6 subunits with a molecular weight of about 21,500. Kushner and Somerville (61) give the CRP molecular weight as 135,000 to 140,000. Their values are consistent with a molecule composed of 6 subunits with a molecular weight of approximately 23,000. The occurrence of a pentameric form in serum

has also been suggested by these authors.

Opinions differ concerning the sedimentation coefficient. For example, Wood, Slater and McCarty (141) give 7.5 S; Kushner and Somerville (61) 6.5-6.7 S.

Gotschlich and Edelman studied the key-position of calcium in the binding properties of C-poly-saccharide and C-reactive protein (37) and produced strong evidence that CRP is bound specifically to primary phosphate groups. In their assay they mentioned that C-polysaccharide contains secondary phosphate groups and felt that the specificity of the protein may be extended to include such groups. They feel that the knowledge of these physical-chemical properties of CRP will be helpful in the investigation of its biological function. Gotschlich and Edelman's finding that dextran sulphate and heparin also precipitate with CRP in the presence of calcium ions should therefore be kept in mind.

Function of CRP. The function of CRP is not known with certainty. In view of Abernethy and Avery's finding that CRP is a protein (1) and of the demonstration of its interaction with pneumococci they, as well as other workers in the field (68) in the early 40s, thought CRP to be an antibody. The key-position of calcium ions in the CRP-C-polysaccharide binding and the evidence that CRP is precipitated between 50-75 % saturation of ammonium sulphate refuted the existence of any relation between CRP and immunoglobulins. The possibility of an intimate relation between CRP and antibodies has, however, occasionally been taken up later. For instance, Wood suggested (138) that the analogue of CRP in rabbits (C_xRP) might be involved in the chain of events leading to antibody formation. As late as 1968 Perez-Miranda and Götz felt that CRP is an antibody (97).

Using direct microscopic examination of the leucocyte

movements in slide cells according to a method described by Martin, Pierce, Middlebrook & Dubos (71) in 1951 Wood (137) demonstrated that CRP stimulates the migration of normal human leucocytes. Later, however, he described the function of CRP as still being enigmatic (139).

In 1962 Hokama, Coleman and Riley (40) studied the effect of CRP on leucocyte phagocytosis of carbonyl iron spherules, Diplococcus pneumoniae and Serratia marcescens. They incubated whole blood and bacteria or iron particles, preincubated with CRP-preparations or control solution. They found that the corpuscles studied were phagocytosed more readily by normal human leucocytes after incubation with C-reactive protein. The leucocyte phagocytosis of bacteria was determined by smearing the phagocytising mixture on slides, staining and counting the visible bacteria or iron spherules ingested per 100 neutrophile leucocytes as well as counting the phagocytically active neutrophile leucocytes. The results obtained were supported by Bakos, Vajda and Weiss' finding (10) that CRP might be ascribed to erythrocytic phagocytosis in polyarthritic patients. These results were, however, not compatible with the opinions of Williams and Quie (1968) (135).

In 1967 Hokama and co-workers reported that C-reactive protein and human hepatic and erythrocytic catalases have immunologically common antigen determinants (44). This group later suggested that appearance of CRP might reflect metabolic changes in the oxidative system, especially in that of catalases (45, 47, 48, 88).

Also other functions have been attributed to CRP. Parker thus assumed that CRP had a "carrier" function (91) - combining with foreign antigen in an unspecific manner and Keitel proposed that CRP was a product of protein degradation (56).

Site of synthesis of CRP. Opinions have differed not

only about the electrophoretic mobility and the function of CRP, but also concerning the site of formation of the protein. The subject has recently been reviewed by Gay and Geiler (33) and, for C_xRP, by Riley, Coleman and Snow (103). According to the most current opinions, CRP or its precursor is produced in the liver (9, 52, 61, 133), or by the inflamed tissue (60), or by the leucocytes (143). The last concept does not agree with our findings(59).

Determination of CRP. The original method for demonstrating CRP by precipitation with soluble C-polysaccharide permitted measurement down to 3 mg/l (73) - 100 mg/l (115). The capsular swelling method demonstrated by Löfström (67) was later introduced as a clinical test by him and by Hedlund (38, 68). This test is said to be more sensitive than the reaction with C-polysaccharide (68, 115). Gál and Miltenyi (31) determined CRP with a hemagglutination method using sheep erythrocytes to which C-polysaccharide had been adsorbed. C-polysaccharide was also used by Abernethy and Francis (2) in their elaboration of a method for demonstrating CRP by subcutaneous injections of the substance. A reaction consisting of delayed erythema, reaching its maximum intensity in 18 to 22 hours was said to be positive.

The preparation of C-polysaccharide from pneumococci was rather laborious, and determination of the non-specific capsular swelling required considerable technical skill. Determination of CRP was therefore not widely used until later elaboration of immunochemical methods and the advent of commercially available antisera. Since then the method devised by Anderson and McCarty (5) has been used most. In this method equal volumes of anti-CRP and the patient's serum are mixed in capillary tubes, and the amount of immunoprecipitate

formed is estimated merely by inspection of the tube. This method is virtually a replica of a method used for determining antibodies against streptococci (123). The concentration of CRP by this method has later usually been expressed as the heights (in mm) of the precipitates formed over night. Though semiquantitative, this method was about 10 times more sensitive than those previously available (115). The main disadvantage of the capillary tube method as it is used all over the world today is the wide variation in anti-CRP titer of commercially available antisera (81), a disadvantage precluding comparison of results obtained on different occasions or at different laboratories.

But there are also several other methods using anti-CRP for determining CRP, suggesting that an ideal method is still lacking. Thus, two methods have been devised by Libretti, Kaplan and Goldin (64), one using an agar precipitation technique according to Oudin and one using a double diffusion in gel method according to Ouchterlony. A disadvantage of the latter is the occurrence of one to three precipitation lines, a phenomenon that has been interpreted as an effect of a heterogeneity of CRP or an interaction of CRP with agar (41), but which has not been found by all using this method (101).

Goldwasser and Roszansky (34) have employed a highly sensitive but laborious immunofluorescent technique and Muschel and Weatherwax (79) a complement fixation test.

Another immunochemical method - with a now commercially available kit - is the latex agglutination test described by Singer, Plotz, Rader and Elster (118). In this method latex particles are coated with anti-CRP. If the patient's serum contains sufficient CRP, the latex particles will agglutinate. A drawback of this method is the so

called prozone phenomenon i.e. the test might be negative when the suspension with anti-CRP coated latex particles are mixed with sera containing very large amounts of CRP. Dilutions of these sera react normally with the latex suspension. According to Braveny and Kopplow (17), these prozone phenomena require a combined test for CRP, using, for instance, the capillary precipitation test (5) for high CRP-concentrations and the latex fixation test for low concentrations.

Tuomioja and colleagues used surfactants containing "carbohydrate - fatty acid esters" for determining C-reactive protein (110, 127, 128, 129, 130, 131, 132). The CRP-combining sites of these products are said to be free hydroxyl groups in the carbohydrate part. The test devised by them is called the APC-test.

The "Penn-test" - a flocculation test in which alcohols of high molecular weight are added to serum (95) and originally designed as a test for cancer was, according to Riley et al. (104), just another way of determining CRP.

In summary, none of the quick tests are precise enough to serve as an indicator of the course of a disease or the effect of a given type of therapy, and the more precise quantitative methods are too slow to keep pace with the quickly changing level of CRP in the inflammatory reaction.

Clinical significance of CRP. The clinical significance of the CRP-determination has been extensively reviewed by Schwarz in 1963 (115) and by Nilsson in 1968 (84). Even Schwarz was able to quote 148 articles on the diagnostic value of CRP in different diseases. It has generally been assumed that CRP is an abnormal protein occurring only in sera from patients with necrotic processes, i.e. bacterial infections, aseptical inflammations or malignant

neoplasms. Most investigators have not been able to demonstrate CRP in viral diseases except hepatitis (38), but this common belief has been disproved by Parker (91), who found CRP in specimens from patients in the acute phase of various viral infections.

Determinations of CRP have been used most in the estimation of the prognosis of myocardial infarction and as a guide in the treatment of rheumatic fever. CRP is said to be a more sensitive indicator than the erythrocyte sedimentation rate, for example. McFarlane et al. recently used serial determinations to check the therapeutic response in children with Burkitt lymphoma (74).

In 1966 Carpenter et al. recommended CRP as an inexpensive screening aid in the examination of large populations for malignant diseases (20). The CRP tests was said to often become positive from 6 months to one year before the patient became aware of any symptoms. Holm and Pompeius (49) have reported that determination of CRP is helpful for distinguishing between renal carcinoma and renal cysts.

Although many publications have emphasised the clinical value of CRP determinations in various diseases, the method has not been used so widely as one might have expected.

NATURAL RESISTANCE

The resistance of the individual to the untoward effect of infections involves two separate systems, the humoral and the cellular defence. Towards the end of the 19th century the opinion of the then predominant German school was that cumulative evidence indicated that the

anti-bacterial activity of serum was sufficient to explain the resistance of the body to the effect of bacterial infections.

The Russian biologist Metchnikoff challenged this concept when he claimed that leucocyte phagocytosis was of major importance in the prevention of a bacterial invasion. A violent controversy arose, especially between von Behring in Germany and Metchnikoff, about the relative importance of cellular and humoral factors in natural resistance.

The gap between the two opinions was bridged by Wright and Douglas (142), who in the beginning of the century demonstrated that phagocytosis is facilitated by substances normally occurring in human serum for which they proposed the collective name opsonins. An intimate interplay between humoral and cellular factors, mobile or immobile, is now generally believed to be responsible for the removal of bacteria from the circulation.

Phagocytosis - the ingestion of small particles by leucocytes or macrophages, including the cells of the reticular endothelial system (RES) - has been extensively reviewed during the last 70 years. Therefore, suffice it here to refer to the classical review by Mudd et al. (77), on phagocytosis and more recent ones by Florey et al. (29), by Brandt (16), by Miler (75) and by Saba (107).

The following studies are confined to leucocyte phagocytosis starting soon after a bacterial invasion. Kvarnstein (62) recognises 5 phases of the phagocytosis, adhesion between particle and cell, cell membrane invagination encircling the particle, fusion with lysosomes, digestion of the ingested material, and elimination of the foreign substance. Only the first two phases in the cycle of phagocytic events will be considered here.

It is known that opsonins render bacteria and especially capsulated bacteria susceptible to phagocytosis. Opsonins are proteins which are bound to bacteria, thereby reducing the strong negative charge of their surfaces and facilitating the leucocyte phagocytosis of the bacteria.

The exact nature of the different opsonins is still hazy, and different classifications have been used. For decades they have been grouped according to their thermostability or thermolability i.e. whether they can tolerate exposure to 56° C for 30 minutes. The largest group of the thermostable opsonins consists of the immunoglobulins, and there is unequivocal evidence that they stimulate phagocytosis by leucocytes or reticular endothelial cells. Antibodies, however, are not usually included in the scope of the term natural resistance, and they are not of any great help the first week after initial exposure to an infectious agent.

The best known representatives of the group of heat labile opsonins are the substances included in the complement system (C1-C9) - a system functionally intimately related to the immunoglobulins. C1 is bound to the antibody fixed on the bacteria. It initiates the whole sequence of events leading to destruction of bacteria. According to a report by Šterzl (120), complement might also act as opsonins without any influence of the immunoglobulins. It has been clearly demonstrated that C1, 4, 2 and C3 are involved in immune adherence and conglutination activities, which are said to be of importance in phagocytosis of bacteria. Lack of sufficient functionally active C3 or C5 has also been incriminated as a cause of recurrent bacterial diseases, but the pathophysiology of this deficiency is not yet clear. The role of the complement system in the defence against the effects

of infection was recently reviewed by Rosen (105).

In an attempt to isolate one of the components of complement Pillemer et al. (99) found a new protein, properdin, not related to the complement system. This protein in conjunction with magnesium ions and complement factors is involved in the destruction of certain gram-negative bacteria. For purification of properdin Zymosan - a high-carbohydrate cell-wall preparation - is added to serum in the presence of magnesium ions. Properdin can be eluted from the properdin/Zymosan complex.

So-called natural antibodies - not connected with the complement system - have also been described by Tullis and Surgenor, who called them phagocytosis promoting factors (126) - one with α another with β electrophoretic mobility. The phagocytosis promoting factor with β mobility is adsorbable with barium sulphate, from which it can be eluted by citrate. According to Surgenor and Tullis, the phagocytosis promoting factors described by them are not identical with properdin (121, 122).

Lysozyme has also sometimes been included among the opsonins. By virtue of its strong basic properties it seems to be adsorbed by the strongly electronegative surfaces of bacteria. Whether this protein plays any role in the maintenance of natural resistance in vivo is not yet known with certainty.

In 1967 Fidalgo and Najjar (25) reported that dog leucocytes bind a specific type of gammaglobulin, isolated by cellulose phosphate chromatography. This fraction stimulated phagocytosis in vitro (26). The fraction was called leucokinin. In 1970 Najjar and Nishioka (80) reported that the whole stimulatory effect of leucokinin can be ascribed to a single peptide fragment liberated by a specific enzyme occurring in the outer

membrane of the neutrophils. This peptide is referred to as tuftsin, and the authors have found 4 people with recurrent infections with very low values of tuftsin in the blood. This phagocytosis stimulating protein differs from the others described above in acting on the leucocytes and not the bacteria to be phagocytised.

Methods for phagocytosis assays. The difficulties and the pitfalls encountered in the investigation of the leucocyte phagocytosis of bacteria in vitro were recently reviewed by Boyden et al. (15) and by Brandt (16). Basically, phagocytosis can be assessed in two ways, viz. by microscopically counting the bacteria phagocytised by observing the phagocytosis directly or after fixation or by colony counting by plating out on blood agar plates a certain amount of the phagocytising mixture. Sometimes only the non-phagocytised bacteria are counted by plating out the supernatant after centrifugation of the phagocytic mixture, and sometimes only the bacteria phagocytised are counted after lysis of the polymorphs. The latter method accounts only for those phagocytised bacteria that are viable at the time of sampling, and neither of these two methods compensate for immunadherence. Another difficulty when counting colony forming units is that many bacteria studied appear in clusters or in relatively long or short chains. It is therefore difficult to know whether a given colony is the result of one or more bacteria.

As already pointed out in the reviews on leucocyte phagocytosis referred to earlier, some authors perform the assay in stationary glass tubes, and this method might include phagocytosis as well as chemotaxis. Others use a "roller drum" procedure by which the glass tube is tumbled end-over-end and which technique is said to include only phagocytosis, but this method might damage the polymorphs and release enzymatically potent proteins. Boyden (15),

however, could not demonstrate any qualitative difference between these two methods.

A common cause of error in phagocytosis assays, pointed out by Boyden, is that opsonins may be adsorbed to the leucocytes if used unwashed. Further, if the bacteria for the phagocytosis assay are cultured on blood agar plates, material of human origin may be adsorbed by the bacteria - material which should be washed off. The mere inclusion of whole serum - diluted or indiluted - in the phagocytising mixture might give rise to very confusing results as activation of different serum components might lead to uncontrolled chain reactions. It might therefore be important to arrange the assay so that the phagocytosis takes place in a physiological salt solutions in which preferably albumin is included for protection of the leucocytes (124). To this solution the specific protein to be assayed may be added. This precaution excludes an uncontrolled chain reaction.

PRESENT INVESTIGATIONS

Purpose and course of present investigations. The primary aim of the present investigation was to find out whether Laurell's electro-immuno assay (62 a) could be used for determine CRP. This required production of anti-CRP, which in turn required isolation of CRP. In the beginning the CRP was isolated according to Hokama et al. (42, 46).

It was observed that on electrophoresis in the presence of calcium ions CRP did not migrate in ordinary agar gels, but was evidently bound to the gel and appeared in the electrophoretic pattern as a very narrow zone immediately cathodally to the application slit (I). In the absence of calcium ions, however, it migrated towards the anode and was then found in the β -region. However,

in certain highly purified agarose preparations in the presence of calcium ions CRP migrated towards the cathode and was found in the γ -region. The electrophoretic mobility of CRP thus varied more widely with the calcium content of the buffer than did that of most other proteins. The electrophoretic behaviour of C-reactive protein and its affinity with calcium were thus analogous to that of prothrombin (32). This similarity suggested that adsorption of CRP to barium sulphate might be useful for purifying CRP, just as it is for purifying prothrombin. This technique had been tried before by Zimmerman and Zack for concentrating CRP (144).

The binding of CRP to agar gels in the presence of calcium ions suggested that CRP was bound to sulphate groups of the gels in a way possibly analogous to the calcium-dependent binding of CRP to phosphate groups of the pneumococcal C-polysaccharide. Since no other protein was known to behave in this way, not even prothrombin, it was thought that adsorption to agar gel in the presence of calcium ions might be useful in the purification of CRP.

Among the plasma proteins adsorbed by barium sulphate Tullis and Surgenor found one fraction to be capable of stimulating phagocytosis by leucocytes (126), which was therefore called the leucocyte-phagocytosis-stimulating factor or the phagocytosis-promoting factor. This factor was shown to migrate electrophoretically like a β -globulin.

CRP was also strongly adsorbed by barium sulphate and was one of the main components of the barium sulphate adsorbable proteins in calcium-free buffer. It was found in the β -region electrophoretically. The finding that barium sulphate adsorbed CRP and the report by Surgenor et al. suggested that CRP and the phagocytosis promoting factor might be identical. This idea was also consistent

with the finding by Hokama, Colman and Riley that carbamyl iron spherules and some bacterial species were phagocytised more rapidly by leucocytes in normal human blood after incubation with preparations of CRP of varying purity (40).

An assay for studying a phagocytosis stimulating effect of CRP on different bacteria was therefore elaborated. The leucocyte phagocytosis of some living pathogenic and non-pathogenic bacteria was shown to be stimulated by CRP in this system (III, IV).

The mechanism of this stimulating effect on phagocytosis was obscure, but presumably required binding of CRP to the bacteria. Such binding of CRP to bacteria was well documented in respect of pneumococci, and it was postulated that CRP might combine also with other bacteria. The aim of one of the present studies (V) was therefore to ascertain whether CRP in whole serum could be adsorbed to living pathogenic gram-positive and gram-negative bacteria, and thereby possibly act as an opsonin.

The primary purpose of the investigations i.e. development of a method for quantitatively determining CRP quickly enough to provide the clinicians with timely accurate information of the CRP in a given case, was achieved by elaborating a conventional electroimmuno assay (I). But this method was not sensitive enough to permit quantitation of CRP in sera obtained from many healthy individuals. A new method was therefore devised (VII). It was based on ¹²⁵I-labelled antiserum, which increased the sensitivity 60-fold.

These methods were later used for calculating the range of variation of CRP concentration in sera from healthy children and adults (VIII).

During the study of CRP in serum in disease it was also observed that CRP could be demonstrated and semi-quantitatively assessed in a standard agarose gel

electrophoretic pattern (VI).

Purification of CRP. (I, II, IV, V). CRP, pure enough for antiserum production, was prepared by the procedure devised by Hokama and Riley (42, 46) with slight modifications which enable batch-wise preparation of several litres at a time without precipitation with ammonium sulphate (I). The starting material was adsorbed on DEAE-cellulose, and CRP was eluted by increasing the sodium chloride concentration. This was followed by preparative electrophoresis (58). The CRP obtained appeared in the electrophoretic pattern as a single narrow band in the γ -zone, trailing from the application slit. The CRP obtained was pure enough for the production of an oligospecific antiserum with anti-CRP. Undesired fractions in the antiserum obtained were easily absorbed off with human serum containing no CRP according to the method devised by Anderson and MacCarty (5). In an Ouchterlony double diffusion system (90) the antiserum obtained after immunisation with this preparation showed a reaction of "identity" with antiserum from Hyland lab. when whole human sera rich in CRP was used as antigen.

A less "laborious" procedure for CRP purification was later elaborated (II). It consisted of two adsorption techniques, originally intended as alternative methods for purifying CRP, but later they proved more efficacious when combined. A pool of sera, ascitic fluid or pleural fluid containing CRP was thoroughly mixed with a loosely packed suspension of barium sulphate. After having been incubated and thoroughly washed the adsorbed proteins were eluted by addition of ammonium sulphate. The CRP of the eluate was precipitated by increasing the ammonium sulphate concentration and collected between 2.0 and 2.5 M (IV). After dialysis against 0.04 M Tris-HCl buffer, pH 7.4, and

equilibration with Tris-buffer containing calcium ions the fractionated protein solution was passed through an agar gel column (IV).CRP was thereby adsorbed to the agar gel, probably to sulphate groups in a way possibly analogous to the formation of calcium-dependent complex between C-reactive protein and the various phosphate groups of pneumococcal C-polysaccharide (37). Chelation of the calcium with EDTA resulted in the formation of pure CRP, as judged from agarose gel electrophoresis and polyacrylamide gel electrophoresis, which was eluted from the column. On crossed immunoelectrophoresis it exhibited the characteristic mobility of CRP. Immunochemical analysis showed a final CRP recovery of about 60-80 %.

The purification of C-reactive protein by adsorption to agar gels seems to be at least comparable to the method of purification by precipitation with pneumococcal C-polysaccharide regarding the specificity and purity of the product obtained. But agar gel is more readily available, which makes the agar gel method simpler and facilitates production of the protein on a large scale.

The choice of agar precipitation was crucial for the second step of the assay. Highly purified agarose preparations, in which CRP migrated electrophoretically in the presence of calcium ions were useless for the adsorption. Only those preparations in which CRP regularly appeared in the electrophoretic pattern as a very narrow zone immediately cathodally to the application slit proved useful.

The sulphate-content did not notably differ between those agar preparations that were found useful and those that were not. This suggests that the sulphate groups occur in different steric positions in different agar preparations.

A third method for preparing CRP (V) was offered by the experiments with in vitro binding of CRP by pneumococci incubated with whole serum. The protein eluted already after a few hours seemed to be pure, as judged from the patterns obtained on agarose gel electrophoresis and crossed immuno-electrophoresis. This third method has an advantage over the above barium sulphate - agar method in that it allows preparation of CRP from serum of most other species studied, while the "combined barium sulphate agar method" is not suitable for preparation of dog CRP, for example. But pneumococci incubated with sera from acutely ill dogs and rabbits effectively adsorbed CRP, which was eluted by chelation of calcium with EDTA. The electrophoretic mobility of CRP thus prepared was found to have the same calcium dependence as human CRP. The relative electrophoretic mobility is demonstrated in Fig. 1.

A similar method was used by Gotschlich (35) for preparation of dog CRP. Differences in the binding of CRP from different species to agar were hardly unexpected. A similar specificity is seen in the binding to C-poly-saccharide. This polysaccharide does not bind rabbit CRP, which is instead bound to the C_x -polysaccharide (6).

Some physical and chemical properties of CRP.(I, II, VI, VIII). The electrophoretic mobility of CRP in agarose gel varies with the presence or absence of calcium or barium ions in the buffer (I, II, VI). In buffers containing calcium or barium CRP appears in the γ -zone, whereas in buffers containing EDTA it migrates with β -proteins. Electrophoresis in the presence of calcium always results in some trailing towards the application slit even if the agarose has been highly purified. In less purified agarose preparations and in agar the CRP persists

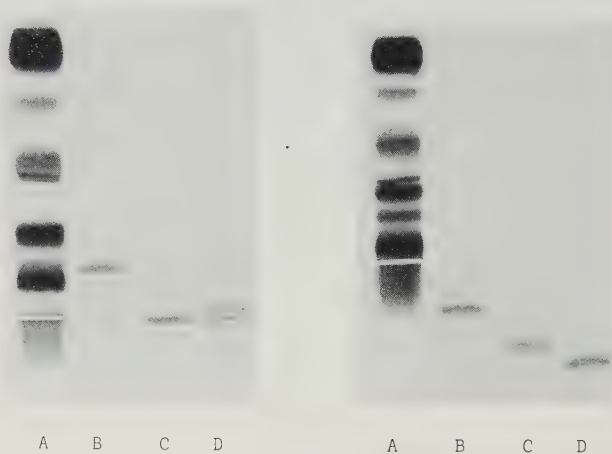


Fig. 1.

Pattern after electrophoresis of eluates with EDTA containing buffer from D. pneumoniae preincubated with a pool of serum from acutely ill humans (B), rabbits (C) and dogs (D). To the left, the electrophoresis was run in 75 mM barbital buffer, pH 8.6, containing 2 mM EDTA and, to the right, in the same buffer in which EDTA was replaced by 2 mM BaCl_2 . The pattern of human plasma is given for reference (A).

at the application slit in the presence of calcium. When calcium was replaced by barium in the buffer used for electrophoresis no trailing occurred. Interaction between CRP and agar or some agarose preparations and the calcium dependent charge of CRP may explain the diversity of the results obtained on electrophoresis in the past.

The electrophoretic mobility of CRP has also been assayed by the immunofixation technique of Alper et al. (3) on 100 consecutive sera from patients at the department of infectious diseases in Malmö. Only those sera which contained at least so much CRP as to produce 1 mm precipitate according to Anderson and McCarty's method (5) were accepted. No variation of the electrophoretic mobility of CRP was observed. The observation by Hornung and Frichi' (51) that the electrophoretic mobility of CRP varied between individual sera could not be confirmed. The material investigated is one third the size of Hornung and Frichi's (51), but large enough to warrant the conclusion that inter-individual differences in the electrophoretic mobility of CRP are uncommon in south Sweden (Vide Fig.2). Genetic variation of CRP remain to be elucidated. But such studies fall outside the scope of the present investigation.

The molecular size of purified CRP was studied with gel filtration and with ultracentrifugation. On gel filtration using Sephadex G-200 and tris-HCl buffer, pH 7.4, containing 0.1 M sodium chloride and 0.001 M calcium chloride, CRP in serum is eluted between the immunoglobulin and albumin peaks. This is in accord with the finding by Nilsson (85). The position is the same when the calcium chloride is replaced by EDTA (disodium salt).

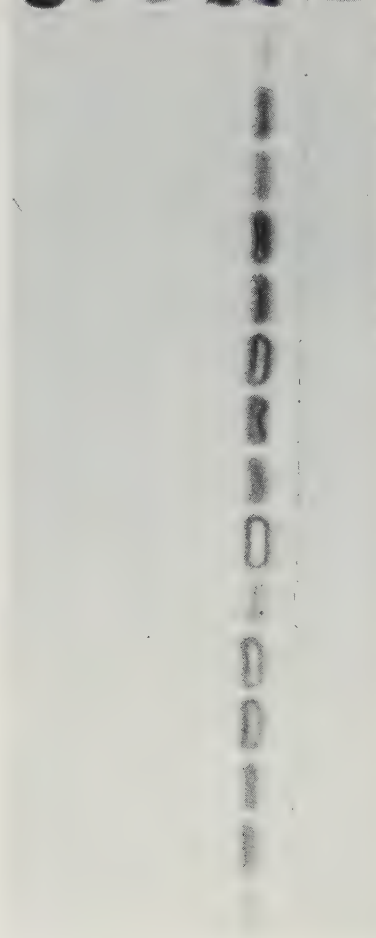


Fig. 2.

Immunofixation (3) of CRP in sera from patients with inflammatory disease by means of anti-CRP. The agarose gel electrophoresis was run in 75 mM barbital buffer, pH 8.6, containing 2 mM EDTA. The pattern of a plasma sample run under identical conditions and stained with Amido black is given for reference.

On ultracentrifugal analysis of a CRP preparation (kindly performed by Ph.D. U.-B. Hansson, Lund) the S-value was found to be about 6.1. Two other S-values of CRP are given in the literature, viz 7.5 (141) and 6.7 - 6.5 S (61). The latter seems to be fairly consistent with ours.

The S-value of CRP and the appearance in the chromatogram on G-200 seem to fit in well with the molecular weight (110,000 - 140,000) reported by Gotschlich and Edelman (36). These observations might indicate that if CRP is associated with lipids (141) or certain blood group substances (43), the association will be unaffected by the presence of calcium or the molecular weight of the complex will not differ essentially from that of pure CRP. Another explanation may be that the affinity between the different parts of the complex is so low that it readily dissociates on gel filtration.

The nitrogen content of pure CRP was found to be 14.9 % (VIII), a value fairly consistent with the findings of McCarty (72).

The absorbance at 280 nm and 1-cm path length of a solution of electrophoretically pure CRP, dialysed against phosphate buffer (0.05 moles/l, pH 8.0, and containing 0.1 moles/l NaCl) whose concentration had been determined immunochemically, is 17.3. This value is fairly good accord with the findings of Wood and McCarty (140).

Using the same CRP solution as for determination of the 280 nm absorbance above the estimation of the relation between absorbance and concentration according to Lowry et al. (66) the regression line was slightly curved (Vide Fig. 3).

Methods of CRP determination. (I, II, IV, VI, VII, VIII).

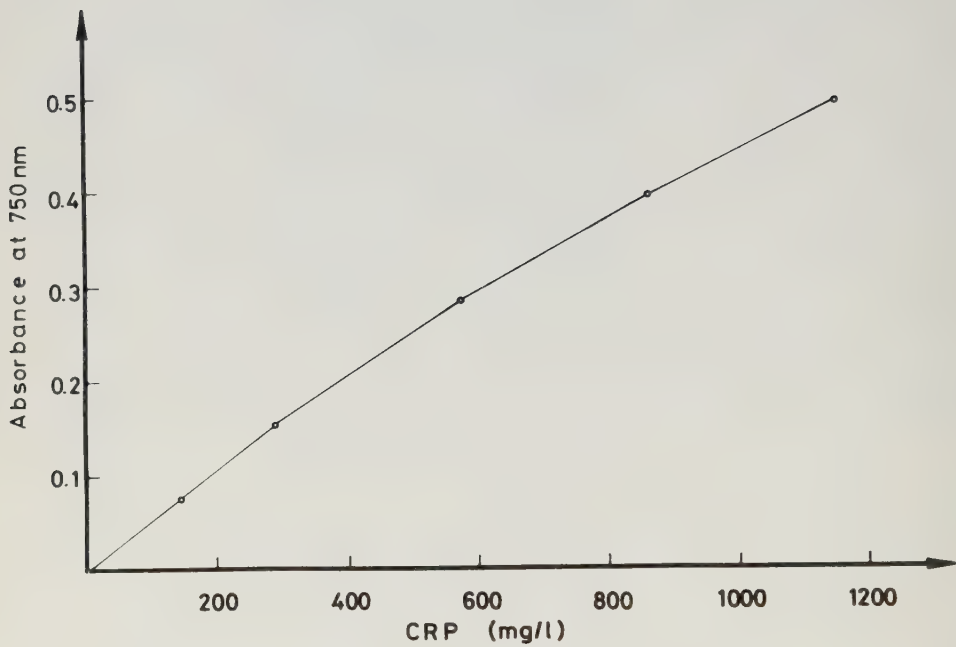


Fig. 3.

The relation between absorbance and concentration of CRP on estimation according to Lowry et al. (66).

The methods available for determining CRP were either semi-quantitative but quick or quantitative, but too laborious to be of any real value in clinical work. The quick, and quantitative electroimmuno assay of Laurell (62 a) was therefore adopted for determining CRP (I). Anti-CRP was obtained by immunising rabbits with pure CRP (II, IV). The antiserum did not require any absorption. In the electroimmuno assay of CRP the electrophoresis is run for 3 to 5 hours depending on the type of agarose used.

If calcium, or preferably barium, is included in the buffers, the precipitates appear on the cathodal side of the well; if EDTA-containing buffers are used, on the anodal side. The precipitates obtained in buffers containing calcium or barium seem to be better outlined than those obtained in the presence of EDTA.

The concentration of CRP in the samples was determined by comparison with a reference serum pool (VIII), which was in turn standardized against pure CRP. For this standardization electrophoretically homogenous CRP was prepared (II, IV) and the CRP-nitrogen content (mg/l) was determined.

The electroimmuno assay permits determination of CRP down to 1 mg/l if amido black is used for staining and to 0.1 mg/l if Coomassie brilliant blue is used (V). This limit allows determination of CRP in all sera with a high normal or abnormally high content.

In an endeavour to demonstrate CRP in healthy blood donors anti-CRP was diluted further until no precipitates were demonstrable, not even by Coomassie brilliant blue. ¹²⁵I-labelled anti-CRP was then used to develop the precipitates. This technique was called radio-electro-immuno assay (REIA) (VII). It is essentially as follows.

Electrophoresis of the samples is run into an agarose gel containing rabbit anti-human-CRP. But the antiserum concentration is one twenty-fifth to one tenth of that in the previous method. After electrophoresis the wet gel is covered with a layer of a ^{125}I -labelled gamma-globulin prepared from rabbit antihuman CRP. Labelled antibodies are then incorporated in the precipitates by additional incorporation of antibodies or by replacing non-labelled antibodies. The gel is then thoroughly washed and the precipitates are revealed by autoradiography. CRP could be demonstrated down to a concentration of 0.01 mg/l.

Even if different methods were used for demonstrating the precipitates at high or low concentrations of CRP, the values obtained on determination of CRP in dilutions of serum varied linearly with the concentration. The precision of the radio-electroimmuno assay expressed as the variation coefficient was 6 % (VII), estimated from duplicates in the same electrophoretic run, and when the anti-CRP concentration was increased to such an extent as to be demonstrable by amido black, the precision of the method was 3.2 % (I).

Abnormally high CRP concentrations in serum or plasma may be recognised from the appearance of the electrophoretic pattern (VI). Thus, on agarose gel electrophoresis CRP is seen as a thin but distinct band in the γ -region if the concentration is above 70 mg/l and the IgG-content is normal.

Concentration of human CRP in normal subjects (VIII).
The presence of CRP in human serum or plasma has long been used as a sensitive indicator of an inflammatory process. But in 1968 Nilsson (86), who used a sensitive immuno-

diffusion method, demonstrated traces of CRP in sera from 50-75 % of blood donors, and Saxstad, Nilsson and Hansson (111) in 17 of 98 apparently healthy infants. Scheiffarth, Pérez-Miranda and Götz (112) corroborated these results immunochemically in concentrated sera from healthy blood donors. These findings strongly suggested that CRP is a constituent of normal plasma - but the lower limit of the normal range remained undefined. Our radio-electroimmuno assay (VII) was developed to study the concentration of CRP and to investigate the CRP for any variation with age and sex.

CRP in a concentration of 0.02 - 13.5 mg/l was regularly demonstrated in sera from apparently healthy children and adults of both sexes. It was found that the distribution of CRP concentration of sera from adults is markedly wide and positively skewed. The logarithmic mean and the mode of distribution did not coincide exactly, indicating that there is no true log distribution. The upper limit of the normal range of the concentration in adults was 4.9 mg/l if defined as the mean plus 2x SD, and 6.1 mg/l if calculated by excluding 2.5 % of the sera with the highest CRP concentration. The values given here agree with those reported by Nilsson (86) and by Saxstad et al. (111). A weak positive correlation was found between CRP concentration and age, an observation also made by Nilsson (86). In contrast with Schumacher (114) and with Kay, Bole and Ledger (55) no difference was found between the group using oral contraceptives and the controls.

It was possible to estimate the CRP concentration in all cord blood samples. This finding refutes the wide belief that CRP is not a protein normally occurring in sera

at birth or later in life (4, 24, 57, 63, 82, 83, 87, 98, 106). The results obtained do not, however, warrant any valid conclusion as to the possibility of a transplacental passage of CRP.

CRP and leucocyte phagocytosis. (III, IV, V). A leucocyte phagocytosis assay was devised, mainly according to Brandt (16). Leucocytes for two phagocytosis assays (with and without CRP) were prepared from blood, obtained with EDTA as anticoagulant, from a single blood donor. The leucocytes were washed with a "physiological salt solution" with EDTA containing 0.5 % serum albumin and with the same solution without EDTA. Serum albumin was included because of its beneficial effect on the viability of leucocytes (124).

The first phagocytosis assay (III) was performed with a micrococcus strain (Gaffkya tetragena), which was cultured on blood agar plates. The bacteria were harvested and washed in salt solution with EDTA and afterwards washed with the same solution without EDTA.

The leucocytes were also washed in salt solution containing EDTA in order to eliminate any adherence of CRP or other substances with a similar tendency to be bound to sulphate or phosphate groups for instance via calcium or barium - substances occurring in human plasma and perhaps also in the material used for making the blood agar plates.

The phagocytosis experiments were always performed as two simultaneous assays (with and without CRP) using the same preparation of leucocytes and bacteria. Before the phagocytosis was started both the leucocytes and the bacteria were incubated separately with CRP or buffer. At the end of experiment droplets of each mixture were spread on microscopic slides, which were dried immediately. The smears

were stained with May-Grünwald-Giemsa and examined microscopically ($\times 1000$).

With the above technique, at most 10 % of the bacteria were phagocytised during incubation (15 min.). The phagocytic activity of the leucocyte was expressed as the number of bacteria phagocytised by the cells during incubation for 15 minutes. 100 leucocytes were examined and divided into 12 groups. The phagocytic activity was regularly higher in the presence than in the absence of CRP. Fig. 4 gives the result of a typical experiment. In the absence of CRP, the majority of the leucocytes had not phagocytised any bacteria, and no leucocyte had phagocytised more than 4 bacteria. After addition of CRP, only 4 % of the cells had not phagocytised any bacteria at all, and some had phagocytised more than 20.

The results could also be given as a total number of bacteria phagocytised by 100 neutrophils. In the experiment given in Fig. 4 22 bacteria were phagocytised in the absence of CRP, compared with more than 1000 in the presence of this protein.

The stimulation of phagocytosis by CRP noted could not, however, be considered a function of this protein until it was known how universal this effect of CRP was, especially on various pathogenic bacteria. The study was therefore extended to include three strains of Diplococcus pneumoniae, Staph. aureus, E. coli and Klebsiella aerogenes, obtained from the routine diagnostic material of the institute of Clinical Bacteriology in this hospital (IV). Diplococcus pneumoniae and Staph. aureus are gram-positive, while the other two are gram-negative. All species are considered pathogenic for human beings.

The phagocytosis assay of these bacteria was slightly modified in that the bacteria were cultured in nutrient broth, instead of on solid blood agar plates, and consequently

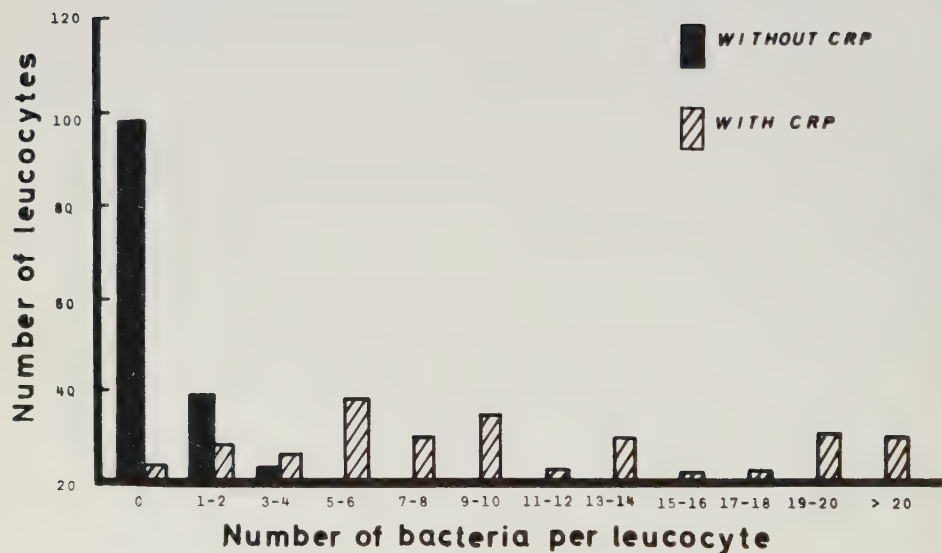


Fig. 4.

Distribution of 100 neutrophil leucocytes according to number of Gaffkya tetragena phagocytosed within 15 minutes in the presence and absence of C-reactive protein.

washing of the bacteria with EDTA was not considered necessary. Only the bacteria were preincubated with CRP for half an hour in these assays and the incubation period for a given bacterial species was varied according to the results of preliminary investigations, i.e. a period was chosen during which sufficient phagocytosis had occurred to permit quantitation. The incubation was stopped by addition of ice-cold salt solution with EDTA and vigorous shaking of the glass tubes to release adsorbed bacteria from the leucocytes and leucocytes from the glass tube wall. Droplets of each mixture were spread on slides, as for Gaffkya tetragena, and dried. The smears were fixed in methanol and stained with methylene blue. The bacteria in at least 100 leucocytes were counted. The ratio between the total number of bacteria (intra- and extracellular) and leucocytes was estimated on the basis of at least 50 polymorphonuclear cells. Simultaneous assays, with and without CRP, were performed.

The mean number of phagocytised bacteria was calculated, and the significance was estimated with the aid of the t-test. Owing to the heterogeneity of variances the number of degrees of freedom was taken restrictively, i.e. the smallest number of leucocytes counted in any of the rows was taken as the number of degrees of freedom.

The phagocytic activity of the leucocytes was expressed as the mean number of bacteria phagocytized per cell. Table I summarises the results obtained with the various species of bacteria.

TABLE I.

Bacterial species	Phag. time (min)	Relation bacteria/leucocytes	No. of leucocytes/mm ³	Mean number of phagocytizing bacteria/leucocytes				Probability	Percentage of phagocytosing leucocytes	
				CRP absent	SD	CRP present	SD		CRP absent	CRP present
<i>occus pneumoniae</i> 3	35	20	44500	0.15	0.5	1.15	2.1	< 0.001	8.0	35.0
<i>umoniae</i> 19	35	18	27800	0.24	0.8	2.63	4.2	< 0.001	9.9	46.5
<i>umoniae</i> 27	35	17	46500	0.38	1.1	2.68	4.1	< 0.001	13.2	60.4
<i>aureus</i> A	30	32	42000	1.22	2.3	3.73	3.9	< 0.001	35.0	76.0
<i>aureus</i> B	30	30	8700	0.32	1.3	0.99	1.5	< 0.001	14.0	43.9
<i>aureus</i> C	30	31	8900	0.28	0.8	1.55	2.4	< 0.001	14.7	46.5
	25	17	66300	0.19	0.6	0.92	1.3	< 0.001	12.1	43.9
	25	9	32500	1.98	2.2	4.21	2.7	< 0.001	67.3	87.1
	25	14	32700	0.55	1.3	2.37	1.8	< 0.001	25.0	83.7
<i>lla aerogenes</i>	40	8	61200	0.55	1.2	1.72	2.1	< 0.001	25.5	64.1
<i>ogenes</i>	40	13	41000	0.31	0.7	0.88	1.2	< 0.001	19.0	51.0
<i>ogenes</i>	40	11	26600	0.08	0.4	0.52	1.1	< 0.001	4.9	51.9

As can be seen in the table, CRP significantly stimulates the phagocytosis not only of the apathogenic Gaffkya tetragena, but also Diplococcus pneumoniae, Staph. aureus, E. coli and Klebsiella aerogenes ($p < 0.001$). The large amount of albumin in the phagocytosis assays excludes the possibility of minor differences in protein concentration between the lot preincubated with CRP and the control having contributed to the stimulating effect of CRP on phagocytosis.

The results obtained were thus in accord with the findings of Hokama et al. (40) and of Bakos et al. (10) and, presuming that the barium sulphate adsorbable phagocytosis promoting factor is identical with CRP, also with the results of Tullis and Surgenor (126).

In assays studying the phagocytosis by the reticulo-endothelial system Norman and Benditt (89) as well as Saba, Filkin and DiLuzio (109) clearly demonstrated that components adsorbable by barium sulphate markedly increase the rate of phagocytosis. This is also true for material soluble at 50 % saturation with ammonium sulphate (100). The components adsorbable by barium sulphate and the proteins soluble at 50 % saturation of ammonium sulphate very probably included CRP. The stimulation of phagocytosis observed in this type of assay might thus be ascribed, at least in part, to an opsonising effect of CRP.

Our finding that CRP stimulates phagocytosis has also very recently been confirmed at Behringwerke by Heide and Seiler (39). They also provide evidence that the effect is dose dependent.

On the other hand, Williams et al. (1968) (135) were not able to show any effect of CRP on phagocytosis. But their phagocytising system as well as the technique used for counting phagocytized bacteria differed from ours. Differences in the preparation and purity of CRP used may

also impair the comparability of the results obtained in the various investigations. In preliminary experiments with whole serum in the present phagocytising system instead of albumin, the rate of phagocytosis was very high, and within less than 10 minutes there was no longer any significant difference in phagocytosis of E. coli and Staph. aureus between the samples with and without CRP. It is therefore possible that Williams et al. (135) may have missed a phagocytosis stimulating effect of CRP because they sampled so late, viz at 30, 60 and 120 minutes after the beginning of the experiment. Like us, Hokama et al. (40) also found such an effect in samples obtained at 10, 15 and 30 minutes.

The use of gelatin, especially in the presence of heparin, in the phagocytising system used by Williams et al. is another point which might be of importance when comparing their results with those reported by Hokama et al. and us. Gelatin used for stabilising colloid in the studies of phagocytosis of the reticulo-endothelial system seems to interfere with the rate of phagocytosis in this system, probably by interaction with the "plasma opsonic system" (27, 53, 78). One might therefore imagine also interaction between gelatin and opsonin in the investigation of phagocytosis by leucocytes. It is therefore quite conceivable that the inability of Williams et al. to demonstrate any phagocytosis-promoting effect of CRP may be ascribed to their technique (personal communication and 136).

Our knowledge of the mechanism of the CRP phagocytosis-promoting effects is still hazy. The capsules of the bacteria are said to inhibit or markedly reduce phagocytosis by leucocytes (22). CRP has long been known to react with the C-polysaccharide of the pneumococcal capsule and to cause swelling of the latter (69). It has also been suggested, although not convincingly demonstrated, that CRP is either absorbed to some extent by E. coli (68), that CRP reacts

with antigens on the surface of gram-negative bacteria (43, 119) or that it alters the bacterial surface of some bacteria (92, 94). It is therefore conceivable that the stimulation of the leucocyte phagocytosis observed may be explained by a binding of CRP to surface structures of the bacteria, altering their surface properties and thereby facilitating phagocytosis by the leucocytes.

The aim of one of the present investigations was to ascertain whether CRP in whole serum could be adsorbed to living pathogenic gram-positive and gram-negative bacteria and whether after the bacteria had been washed several times, the CRP could be recovered from them on chelation of the calcium by EDTA.

CRP was demonstrated in such EDTA-elutes from all the bacterial species studied i.e. Gaffkya tetragena, Staph. aureus, Dipl. pneumoniae, E. coli and Klebsiella aerogenes, but the total amount adsorbed and recovered varied considerably with the bacterial species. E. coli thus adsorbed only 1 %, and Staph. aureus only 0.01 %, of the amount of CRP adsorbed by the same number of Dipl. pneumoniae (V). But the adsorption of CRP by pneumococci as well as by other bacteria was not unspecific. The eluates from E. coli consisted to about 80 % of a β -globulin, which crossed immunoelectrophoresis identified as CRP. Judging from results obtained with the same techniques, CRP constituted at least 95 % of the protein in the eluates from the pneumococci.

The amount of the serum CRP adsorbed by some bacteria was small and was therefore difficult to calculate from the difference between the CRP concentration before and after the addition of bacteria to the serum. The amount of CRP that was adsorbed by the cells was therefore measured as the amount that remained adsorbed after the cells had been washed in buffer containing calcium. It was specifically

eluted by the buffer containing EDTA. But the amount of CRP eluted from the cells is a minimum value for the amount adsorbed, since any CRP adsorbed and not eluted by EDTA will not be included in the calculation.

C-polysaccharide has been demonstrated in extracts of such different organisms as fungi (12, 65, 96) and liver flukes (19). CRP has also been shown to interact with phosphate groups (37, 54, 102, 134) and sulphate groups (144, II) - structures present in most bacterial envelopes. It is therefore not surprising that CRP can be shown to be bound also by bacteria other than pneumococci.

The large difference in CRP binding capacity between the different types of cells may perhaps be explained by difference in amount and type of receptor groups on the cell surface, which may in turn be due partly to the conditions under which the bacteria are cultured (65).

Judging from the results reported, CRP is absorbed by different bacteria and CRP might be regarded as an opsonin. An absorption with different bacteria is known to remove phagocytosis-stimulating factors from serum (116, 117). This depletion of opsonic activity might be ascribed, at least partially, to CRP adsorption to the bacteria.

CRP is very weakly bound to Zymosan under the experimental conditions used (V). But this does not warrant any conclusion about a possible relationship between CRP and properdin. On the other hand, Tullis and Surgenor were of the opinion that these two proteins are not identical (126). Further, the above-mentioned phagocytosis does not require the presence of complement which properdin does.

Summing up, the present investigations have shown that CRP has a phagocytosis-stimulating effect when tested in a leucocyte phagocytising system using different apathogenic

as well as pathogenic bacteria. It has also been shown that CRP in whole serum is more or less specifically bound to the same species of bacteria. It is therefore conceivable that the binding of CRP to bacterial surfaces changes the properties of the latter. Protein coated bacteria may be more readily phagocytised than uncoated ones, a difference that might help to explain the stimulating effect of CRP on phagocytosis.

GENERAL DISCUSSION

The phagocytosis-stimulating effect of CRP might be explained by opsonisation of the bacteria. Wood's finding in 1951 (137) that CRP stimulates movement of leucocytes suggests another or supplementary explanation that CRP might have a general stimulating effect on white blood cells. The CRP-stimulating effect on leucocytes might be chemotactic - and the possibility of chemotaxis cannot be excluded in the phagocytising system used.

Preliminary results in an assay of the chemotactic effect of CRP on leucocytes with a modified Boyden's technique (14) indicate a positive chemotaxis. Our knowledge of the substances and reactions behind chemotaxis is still vague. In vitro investigations of the chemotactic effect of bacteria on amebae have shown that cyclic 3', 5'- Adenosine monophosphate (cyclic AMP) is involved (13). Being a link in many physiological events the substance is now receiving extensive attention and has to be considered in future work on the relation between CRP and its chemotactic effect.

Depletion of opsonic activity during the first hours after a major surgical operation (108) and after severe burns (113) is well documented. CRP is said to be found in necrotic tissue (56, 60) and the marked increase in CRP concentration after a surgical trauma, for example, is not

demonstrable until 4-8 hours after the operation (7). The depletion of opsonic activity and low CRP concentration seem to occur simultaneously. In view of our present knowledge about opsonins, determination of CRP concentration the very first hours after an induced inflammation or surgical trauma would be of great interest. We might be able to reveal a concomitant decrease in CRP concentration and resistance to bacteria shortly after the trauma, as is known to occur in certain animals (93).

The ability of CRP to be bound to phosphate groups of polysaccharides is documented (37). Such substances normally occur in the body, especially in joint cartilage. It is a commonplace that leucocytes have difficulties in coping with joint infections. The decreased natural resistance of joints might be explained by a decreased CRP activity. This possibility has, not yet been tested experimentally.

Defective opsonisation has recently been reviewed by Douglas (23). As pointed out by him defective opsonisation leading to intercurrent bacterial inflammations has been reported, and these defects may sometimes be controlled by treatment with normal plasma (76). The defect is supposed not to involve the complement system. It would be interesting to measure the concentration of CRP in serum from such patients and their family members. If CRP is found in a low concentration during infection, it might be worth while to try administration of CRP.

We have found "CRP deficiency" in a 2 year old girl during an attack of acute otitis media (III). This girl had by that time already had the condition 26 times. The girl had been adopted by Swedish parents and her biological parents were living abroad and could not be traced. Another case has been reported by Belfrage and Lundh (11).

But before treating deficient human beings with CRP the substance must first be studied for any adverse effects. Such experiments have already been started. Prof. Schwick at Behringwerke AG in Marburg/Lahn has kindly provided one preparation of CRP and albumin and, as a control, one consisting of only albumin. In preliminary trials pneumococci incubated with respectively this CRP albumin solution and the pure albumin solution have been injected intraperitoneally into mice. Within 4 days 7 out of 10 mice in the "albumin group" died and pneumococci could be cultured from heart blood. One of 10 in the "CRP - albumin group" died during the same observation period - actually within 17 hours of injection - and no pneumococci could be cultured from the heart blood. A large hematoma was found in the abdominal wall in this mouse. These findings suggest the desired effect of CRP on highly pathogenic microorganisms in vivo.

In the study of CRP bound by different pathogenic bacteria we had the impression that virulent pneumococci - that had been passed through a mouse - bound more CRP than the same strain after being subcultured several times. Also freshly isolated E. coli bound more CRP than other laboratory adapted strains of the same species. It is not yet known whether virulence and ability to adsorb CRP of the bacteria are interrelated. As far as I know, there is no generally accepted way to increase virulence of other bacteria than pneumococci. But if such an interrelationship could be demonstrated, it would further strengthen the assumption that satisfactory phagocytosis requires a good supply of CRP early in bacterial infections. It would also appear that the degree of phagocytosis in various systems varies with the virulence of the bacteria used. Thus, the connection between the virulence of bacteria, human resistance and CRP needs to be elucidated further.

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